DNA METHYLATION IN IMPRINTED GENES IGF2 AND GNASXL IS ASSOCIATED WITH PRENATAL MATERNAL STRESS

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List of Abbreviations

AHO	Albright's hereditary osteodystrophy
AUC	Area under the curve
CpG	Cytosine – guanine dinucleotide
DF	Degrees of freedom
DMR	Differentially methylated region
DOHaD	Developmental origins of health and disease
EDS	Edinburgh depression scale
GNAS	Guanine nucleotide binding protein, alpha stimulating
GNASXL	GNAS extra-large
HPA	Hypothalamic-pituitary-adrenal
ICR	Imprinting control region
IGF2	Insulin-like growth factor
IGF2AS	IGF2 anti-sense
LASSO	Least absolute shrinkage and selection operator
PELS	Prenatal early life stress
PHP	Pseudohypoparathyroidism
PRAQ	Pregnancy related anxiety questionnaire
SD	Standard deviation

ABSTRACT

Epigenetic regulation of imprinted genes during embryonic development is influenced by the prenatal environment. Our aim was to examine the effect of maternal emotional stress and cortisol levels during pregnancy on methylation of imprinted genes *IGF2* and *GNASXL* using umbilical cord blood DNA. Maternal depressed mood (Edinburgh Depression Scale; EDS), pregnancy-related anxiety (PRAQ) and cortisol day profiles were assessed throughout pregnancy. At birth, a cord blood sample (n = 80) was taken to study DNA methylation of IGF2 DMR0, IGF2AS and GNASXL using Sequenom EpiTYPER. Linear mixed models were used to examine the relationship between DNA methylation and maternal stress, while correcting for confounders. We also studied the association of DNA methylation with the child ponderal index at birth. We found a CpG-specific association of PRAQ subscales with IGF2 DMR0 (CpG5, $p<0.0001$) and GNASXL (CpG11, $p=0.0003$), while IGF2AS was associated with maternal EDS scores (CpG33, $p=0.0003$) and cortisol levels (CpG33, $p=0.0006$; CpG37-38, $p=0.0005$). However, there was no association of methylation with ponderal index at birth.

In conclusion, maternal stress during pregnancy, as defined by cortisol measurements, EDS and PRAQ scores, is associated with DNA methylation of imprinted genes IGF2 and GNASXL. Our results provide further evidence that prenatal adversity can influence imprinted gene methylation, although future studies are needed to unravel the exact mechanisms.

INTRODUCTION

The prenatal and early postnatal environment can influence an individual's health and disease susceptibility. Epigenetics has been proposed as one of the mechanisms of this Developmental Origins of Health and Disease (DOHaD) hypothesis (Waterland & Michels, 2007). For instance, the prenatal environment can influence fetal DNA methylation marks, including the ones associated to imprinted loci. These are a small percentage of all genes in our genome that are either maternally or paternally silenced and hence are only expressed from one allele (Constancia *et al.*, 1998, Reik & Walter, 2001, Tilghman, 1999). Most imprinted genes are located in clusters across the genome which are epigenetically regulated by imprinting control regions (ICR) (Reik & Walter, 2001).

A number of studies have shown an influence of prenatal environment in determining the methylation pattern of imprinted loci (Murphy *et al.*, 2012a, Steegers-Theunissen *et al.*, 2009). Maternal exposure to famine during pregnancy brings about DNA methylation changes in imprinted genes in the offspring that persist throughout life, as well as changes in other genes involved in growth and metabolism which may increase the risk for pathology such as cardiovascular disease (Heijmans *et al.*, 2008, Tobi *et al.*, 2014, Tobi *et al.*, 2009). Furthermore, parental obesity as well as maternal gestational diabetes was recently shown to influence a newborn's DNA methylation at certain imprinted genes (Chen *et al.*, 2014, Soubry *et al.*, 2015, Soubry *et al.*, 2013).

In addition to maternal diet and health, maternal stress including depression is another interesting factor that can influence fetal epigenetic marks (Szyf, 2013). Maternal depression and anxiety during pregnancy can have a widespread impact on DNA methylation of neonates (Non *et al.*, 2014). Several other studies have provided evidence of epigenetic changes in hypothalamic-pituitary-adrenal (HPA) axis related genes of children prenatally exposed to maternal stress. Especially the glucocorticoid receptor gene was found to be hypermethylated in association with stress, often leading to an overactive HPA axis (Glover

et al., 2010, Turecki & Meaney, 2014). Recent studies provide evidence that maternal depression or stress during pregnancy can influence birth weight as well as newborn methylation of imprinted genes, such as *MEG3* and *MEST* (Liu *et al.*, 2012, Vidal *et al.*, 2014).

The insulin-like growth factor gene (IGF2) and the genomic locus encoding the guanine nucleotide binding protein (G Protein) alpha stimulating (GNAS) are among the most complex and well characterized imprinted genes, both in mice and in humans (Chao & D'amore, 2008, Plagge *et al.*, 2004, Turan & Bastepe, 2013).

IGF2 is located in a cluster of imprinted genes at chromosome 11p15.5, comprising several differentially methylated regions (DMR). Paternal expression of IGF2 is maintained by methylation at the ICR located between IGF2 and H19. Paternally methylated IGF2 DMR0 is located at the beginning of the IGF2 gene and is involved in prenatal development. IGF2AS is transcribed from the complementary DNA strand. Although the IGF2AS promoter lies within a CpG island in humans, in mice it is located in a regulatory region, 'DMR1'.

The 20q13.3 region contains the GNAS complex locus which has four alternative first exons that splice onto exon 2 to generate different transcripts. Maternal, paternal or biallelic expression of the transcripts is regulated by the three DMRs. For instance, the extra-large (XL) exon is only paternally expressed to generate the XLalphas transcript.

These two genes are involved in specific gene networks that control fetal growth and development (Varrault *et al.*, 2006). Many imprinted genes and mechanisms are conserved in mammals and disruption is often associated with abnormal growth, development and even behavior. Indeed, this indicates that proper imprinting is crucial in early life and it illustrates the importance of the prenatal environment in development of health and disease (Hitchins & Moore, 2004).

The aim of this study was to investigate DNA methylation at IGF2 and GNASXL as potential responders to maternal depressive state, pregnancy-related anxiety and cortisol levels and its role in influencing ponderal index at birth.

MATERIALS AND METHODS

Subjects

Women were included in our Prenatal Early Life Stress (PELS) study in their first trimester of pregnancy at about 6 to 12 weeks of gestation. The study was conducted in the University Hospitals of Leuven, Belgium. The mean age was 30.0 years (SD = 3.94) and for about half of the women, this was their first pregnancy (54.2%). At each trimester, maternal mood and cortisol levels were assessed. For 80 of the mothers, we were able to collect a blood sample from the umbilical cord at delivery. About half of the babies (48.8%) were boys.

Most women (>70%) were highly educated and had a high socioeconomic status. All mothers took folate supplements, eliminating the need to correct for this potentially influencing factor (Steegers-Theunissen *et al.*, 2009).

This study has been approved by the ethical committee of the University Hospitals of Leuven Belgium (n° S51757). Written informed consents were obtained from all participants.

More information and an elaborate description of the PELS study population has been published previously (Hompes *et al.*, 2012).

Prenatal environment

Maternal mood was evaluated at each trimester of pregnancy, using three main variables. The Edinburgh Depression Scale (EDS) was used to screen for depressive symptoms and is validated for the perinatal period (Bergink *et al.*, 2011). Anxiety related to pregnancy-specific issues was examined using the Pregnancy-Related Anxiety Questionnaire (PRAQ) (Van Den Bergh *et al.*, 2005). More specifically, we used the revised version which was validated by Huizink et al, comprising three subscales examining 1. maternal anxiety related to changes in one's appearance ('PRAQ-change'), 2. fear of the act of giving birth to the baby ('PRAQ-

delivery') and 3. concerns about the baby's health ('PRAQ-integrity') (Huizink *et al.*, 2004). Since the three subscales are so distinct and uncorrelated, we analyzed their effects separately instead of as one PRAQ variable.

General anxiety was assessed using the Spielberger State-Trait Anxiety Inventory (STAI) (Spielberger, 1983).

Additionally, maternal cortisol was measured by cortisol diurnal response in saliva, using the High Sensitivity Salivary Cortisol Enzyme Immunoassay Kit (Salimetrics, Suffolk, UK). The area under the curve was calculated using the trapezoidal rule.

EDS, PRAQ and STAI were administered in parallel with cortisol level measurements at each trimester and average values across the entire prenatal period were calculated for further analyses.

DNA methylation analyses

The IGF2 DMR0, IGF2AS and GNASXL amplicons used were previously described (Heijmans *et al.*, 2007, Izzi *et al.*, 2010, Izzi *et al.*, 2012b).

A schematic overview of the amplicons is represented in Figure 1. Additional amplicon information, including primers and sequence can be found in Supplementary Table S1. IGF2 DMR0 CpG units were numbered analogously to Heijmans *et al.* (2008).

For each cord blood sample, DNA was extracted from peripheral blood mononuclear cells and 1 µg of DNA was subjected to bisulfite treatment using the MethylDetector kit (Active Motif, Carlsbad, CA, USA). For the conversion reaction we used a long incubation protocol as proposed by Izzi *et al.* (2014). Bisulfite treated DNA samples were subjected to PCR for amplification of the IGF2DMR0, IGF2AS and GNASXL amplicons (Table S1). Next, DNA methylation was analyzed according to the Sequenom MassARRAY T-cleavage protocol (Sequenom, San Diego, CA, USA) (Coolen *et al.*, 2007, Ehrich *et al.*, 2005, Van Den Boom &

Ehrich, 2009). The EpiTYPER software (v1.0 build 1.0.5, Sequenom) was used to visualize the data.

Each amplicon was amplified and analyzed in triplicate. DNA methylation values with SD between replicates of more than 10% were excluded for further analyses as described (Hompes *et al.*, 2013, Izzi *et al.*, 2010, Rochtus *et al.*, 2015), and only CpG units with a success rate of 60% or more were included for further analyses.

Statistical analysis

To examine the effect of maternal EDS scores, PRAQ subscale scores and cortisol levels on methylation measurements in cord blood, a linear mixed model was estimated for each amplicon (IGF2 DMR0, IGF2AS and GNASXL) separately. STAI measurements were not included in the analyses, since they were highly collinear with EDS scores (Spearman R^2=0.747) and did not significantly contribute to the results. In the linear mixed model, measures of DNA methylation at different CpG units (level 1) were nested within subjects (level 2) (Verbeke & Molenberghs, 2000). Mixed models have the advantage of using all available data, can properly account for correlations of several methylation measures on the same subject, and have great modeling flexibility. We evaluated following variables as potential confounders: maternal age, gender of the baby, alcohol during pregnancy, smoking during pregnancy, maternal weight before conception, maternal length, method of birth, gestational age at birth, cord blood pH, parity and gravidity. Ultimately, gender of the baby, smoking during pregnancy and gestational age were included in our statistical model.

The following linear mixed model was estimated:

$$Y_{ij} = (\beta_0 + U_i) + \sum_{p=1}^{P} \beta_p X_{pi} + \sum_{c=1}^{C} \beta_c X_{ci} + \varepsilon_{ij}$$

where Y_{ij} is DNA methylation level in either DMRO, IGF2, or GNASXL for Subject i at CpG j, β_0 is an overall intercept, U_i is a random effect for Subject i that is assumed to be normally

distributed with $U_i \sim N(0,\sigma_u^2)$, X_{pi} is the score of Subject i on the P predictors of interest, respectively, EDS score, PRAQ subscale scores, cortisol levels and dummy variables representing CpGs and their interaction with the previous mentioned variables. Further, β_p are the corresponding fixed regression coefficients. In addition, X_{ci} is the score of Subject i on covariate c of the C covariates of interest, being smoking status of mother during pregnancy, gestational age, and gender of the baby, and their interactions with dummy variables representing CpG units. β_c are the corresponding fixed regression coefficients. Finally, ε_{ij} is an error-term that is assumed to be normally distributed with $\varepsilon_{ij} \sim N(0,\sigma_\varepsilon^2)$. All tests were two tailed. A p-value of <0.05 was considered statistically significant. When the CpG x predictor interaction (e.g., CpG x PRAQ delivery) was significant, post-hoc tests were performed to evaluate at which CpG units the slopes were significantly different from zero. For these post-hoc tests, Holm's correction for multiple testing was applied. All analyses were performed in SAS 9.2. (SAS Institute ind., Cary, NC).

To predict baby's ponderal index at birth based on CpG methylation, we first examined the three amplicons separately. For DMR0 and IGF2AS, we performed multiple regression with ponderal index at birth as criterion and methylation at the different CpG units as predictors. For GNASXL, we used Least Absolute Shrinkage and Selection Operator (LASSO) regression since methylation at the different CpG's were highly correlated, implying multicollinearity problems in ordinary multiple regression (Hastie *et al.*, 2009, Tibshirani, 1996). LASSO is a regularization procedure that shrinks regression coefficients toward zero, and is equivalent to maximum penalized likelihood estimation with a penalty function that is proportional to the sum of the absolute values of the regression coefficients. The degree of shrinkage of the parameters towards zero depends on a tuning parameter that balances the trade-off between model fitting and model sparsity; selection of the tuning parameter was based on minimizing of the mean prediction error using cross-validation. LASSO is helpful to select relevant predictors from a large set of predictors and to deal with multicollinearity data.

Finally, LASSO regression was also used to predict ponderal index at birth on the basis of all CpG units of the three amplicons together.

RESULTS

Prenatal environment and DNA methylation of IGF2 DMR0, IGF2AS and GNASXL

A schematic overview of the genes, amplicons and CpG units examined can be found in Fig. S1 and Table S1. Average DNA methylation per CpG unit for each amplicon is reported in Table S2. The IGF2AS amplicon had generally low DNA methylation, with CpG units mostly below 10% methylation. On the other hand, IGF2 DMR0 and GNASXL had a methylation of about 50% and 30%, respectively. Notably, CpG units within the GNASXL amplicon have a higher inter-location correlation as compared to the IGF2 amplicons. More in particular, the mean of all 36 pairwise correlations among GNASXL CpG units is 0.77 (SD=0.15).

Using separate linear mixed models for each amplicon, the effect of maternal stress measurements on DNA methylation in cord blood was examined. Several variables were evaluated for a potentially confounding influence (see Methods section) and ultimately we corrected for smoking during pregnancy, gestational age and gender of the baby.

IGF2 DMR0

Analysis for IGF2 DMR0 (see Table 1) revealed a significant association with the PRAQ-integrity, dependent on CpG unit ($p=0.0012$). Post-hoc tests were performed to evaluate at which CpG unit(s) the correlation with PRAQ-integrity was significantly different from zero. As can be seen in Figure 1, this analysis showed that IGF2 DMR0 DNA methylation significantly decreases with increasing PRAQ-integrity at CpG5 ($p<0.0001$). The regression coefficient is -0.05809, indicating that for each point increase in PRAQ-delivery score, DNA methylation decreases by 5.8 % (Table 1). At the other CpG units – CpG1, CpG2-3, and CpG4 – there is no significant relation between PRAQ-integrity and methylation. The findings remained significant after correction for multiple testing using Holm's correction for multiple testing.

No association was found between maternal EDS or cortisol levels and IGF2 DMR0 methylation.

IGF2AS

With respect to IGF2AS methylation, we found significant associations with EDS (p=0.0415) and cortisol (p=0.0019), dependent on CpG site. Post-hoc analyses revealed that increased maternal EDS scores throughout pregnancy are associated with a decreased DNA methylation at IGF2AS CpG33 (p=0.0003). The regression coefficient for this relationship is -0.00556, indicating that for 1 point increase in EDS, DNA methylation decreases by 0.56 % (Fig. 2a, Table 2). At the other IGF2AS CpG units, no significant association was found between EDS and DNA methylation.

Furthermore, maternal cortisol levels during pregnancy were significantly associated with IGF2AS methylation at two CpG units (Figure 2b). At CpG33, cortisol has a positive regression coefficient (β=0.000287, p=0.0006), indicating that for 100 units increase in cortisol (µg/dL), CpG37-38 DNA methylation decreased by 2.9 %, while the regression coefficient for CpG37-38 was -0.00026 (p=0.0005), indicating that for 100 units increase in cortisol (µg/dL), CpG37-38 DNA methylation decreased by 2.6 %.

All relationships remained significant after Holm's correction.

Note that we did not find evidence that the effect of maternal emotional stress is mediated by maternal cortisol levels during pregnancy, as there was no significant relation between emotional stress, the presumed causal variable, and cortisol levels, the mediator (p=0.30)

GNASXL

GNASXL methylation was also associated with maternal PRAQ-delivery (p=0.001). Further analysis shows significance at CpG11, CpG13-14, CpG18-19, CpG20-21-22, CpG25 and CpG27 (see Table 3). All associations were positive. The largest regression coefficient was

found for CpG11 (β=0.02619, p=0.0003), indicating that an increase of one unit on the PRAQ delivery score entails an increase of 2.6% methylation. Following correction for multiple testing, only CpG11 remains significantly associated with PRAQ-delivery (Fig. 3). Note that methylation was highly correlated at the different CpG units in this amplicon. As a result, similar effects are seen at the other CpG units mentioned above.

Ponderal index at birth and DNA methylation of IGF2 and GNASXL

The mean (±SD) birth weight was 3.369 kg (± 0.517), ranging from 1.640 to 4.450 kg. The mean ponderal index at birth was 26.1 kg/m³ (± 2.3). As an exploratory analysis, we examined the association of IGF2 DMR0, IGF2AS and GNASXL DNA methylation at the different CpG units with the baby's ponderal index at birth. Multiple regression analysis did not reveal any association of IGF2 DMR0 ($F(4,34)=0.38$, $p=0.8219$), nor IGF2AS ($F(11,32)=1.05$, $p=0.4289$) with ponderal index at birth. In contrast to IGF2 DMR0 and IGF2AS, GNASXL CpG units are highly intercorrelated. To avoid these multicollinearity problems, we used LASSO regression for GNASXL, instead of ordinary multiple regression. None of the GNASXL CpG units were significantly associated with child ponderal index at birth. Including all three amplicons in the LASSO analysis also did not reveal any significant associations.

DISCUSSION

Critical periods of development early in life, such as the perinatal period, are sensitive to changes in the environment. Although the embryo's genome is set, it can be programmed by environmental influences in a way that is either adaptive or maladaptive. Moreover, these effects can prepare the individual to cope with adversity or even predispose for pathologies such as metabolic syndrome, cardiovascular disorders or affective disorders (Bale *et al.*, 2010, Van Den Bergh, 2011, Waterland & Michels, 2007).

The existence of congenital imprinting disorders that often comprise variable degree of growth, metabolism and behavior abnormalities, make imprinted genes highly relevant as potential recorders of prenatal environmental stimuli with consequences on fetal phenotype. In the current study, we assess DNA methylation changes in three imprinted genes, instead of examining actual loss or gain of imprinting.

Recently, there has been a growing interest in examining the effect of prenatal environment on epigenetic patterns of imprinted genes. For instance, influences of maternal cigarette smoking on fetal DNA methylation are important and can be tissue- and gender-specific (Murphy *et al.*, 2012a, Nielsen *et al.*, 2014). Maternal lead exposure is another influencing environmental factor in this context (Goodrich *et al.*, 2015). Similarly, maternal health, i.e. obesity or gestational diabetes was shown to alter fetal DNA methylation at imprinted genes (Chen *et al.*, 2014, Soubry *et al.*, 2015).

In our current study we found an association of IGF2 DMR0, IGF2AS and GNASXL DNA methylation in cord blood with maternal stress during pregnancy.

When comparing methylation values per CpG unit in each region, our measurements seem comparable to those previously published (Izzi *et al.*, 2010, Izzi *et al.*, 2012b, Tobi *et al.*, 2012) and our results seem to be consistent with findings of other studies examining the impact of prenatal environment on DNA methylation of imprinted genes.

For IGF2 DMR0 we observed a link with maternal pregnancy-related anxiety regarding the health of the baby, whereas fetal DNA methylation of the IGF2AS amplicon was associated with depression scale scores and cortisol levels.

Both *IGF2* and *IGF2AS* genes are located in the *IGF2* gene cluster which is regulated by an imprinting control region and two DMRs. Changes in the epigenetic marks of these regions may cause changes in gene expression and even pathology. For instance, *IGF2* has been widely studied as an oncogene and altered methylation of its DMRs have been associated with colorectal and breast cancer (Baba *et al.*, 2010, Barrow *et al.*, 2015). In mice, the promoter of *Igf2as* (also referred to as *PEG8*) is located in a DMR ('DMR1'). Although such a DMR is not present in humans, methylation changes in this region have been observed in Albright's hereditary osteodystrophy (AHO)-like patients with short stature (Izzi *et al.*, 2012b). Not much is known about the function of this *IGF2AS* gene, although its DNA methylation has been suggested to play a role in early life stress (Bick *et al.*, 2012).

Many studies focus on the *IGF2* gene, of which the IGF2 DMR0 region is the most studied in DNA methylation studies. Widely known is its association with low maternal caloric intake during a period of famine described in the Dutch Hunger Winter Study (Heijmans *et al.*, 2008). Here, Heijmans *et al.* (2008) report lower IGF2 DMR0 methylation in children that were prenatally exposed to famine during the Dutch Hunger Winter. They indicate that there may be an additional contribution of other factors, such as maternal emotional stress. Indeed, in concordance with this study, we provide evidence of the influence of pregnancy-related anxiety on IGF2 DMR0 methylation as well. Pregnancy-related anxiety, more specifically anxiety related to the baby's health, can be a measure of profound maternal stress. Therefore, both effects may be complementary in the programming of fetal DNA methylation.

DNA methylation in the IGF2AS amplicon was associated with maternal cortisol levels during pregnancy, independently of other maternal stress measures. Conversely, this suggests that

the mechanism for the effect that we find of maternal emotional stress is not mediated by maternal cortisol levels, as we suggested in a previously published paper regarding the glucocorticoid receptor gene (Hompes *et al.*, 2013). Although cortisol promotes maturation of fetal organs, it has also been suggested to play a role in prenatal programming and may adversely influence development (Davis & Sandman, 2010).

GNAS methylation changes have been associated with Pseudohypoparathyroidism (PHP) and AHO features (Izzi *et al.*, 2012a, Izzi *et al.*, 2012c). Additionally, paternally inherited mutations in the *GNAS* gene and loss of expression of *GNASXL* give rise to more pronounced intrauterine growth retardation as compared to mutations on the maternal allele (Richard et al. 2013). In mice, *Gnasxl* is associated with absent suckling, defective glucose metabolism and leanness (Peters, 2014, Plagge *et al.*, 2004).

In literature, studies suggest an association of fetal growth with methylation changes in IGF2 DMR0 as well as GNASXL (Bouwland-Both *et al.*, 2013, Brehin *et al.*, 2015, Izzi *et al.*, 2012b). Remarkably, we found no correlation between DNA methylation in either region and ponderal index at birth.

There may be a few explanations for the lack of significant findings regarding ponderal index at birth in this study. First, since regression excludes cases listwise, the analysis was carried out on only a subset of the study sample, including 33 out of 80 subjects. Second, it may be due to the fact that the weight measures of our study population are within the normal ranges. A study population with more low birth weight individuals might have provided more evidence for these associations. However, an association between IGF2 methylation and birth weight or ponderal index is not always found (Burris *et al.*, 2013, Tobi *et al.*, 2011). Further, IGF2 loss-of-imprinting has been demonstrated in phenotypically healthy infants (Rancourt *et al.*, 2013).

Currently, we cannot determine if these babies exposed to prenatal stress have an increased predisposition to metabolic or other diseases later in life including psychiatric disorders, as

described by several studies (Entringer *et al.*, 2010, Harrison *et al.*, 2003, Pidsley *et al.*, 2010, Wadhwa, 2005).

All observed findings in this study were CpG-specific, which means that there was no main effect for the whole amplicon region, but only certain CpG units in the amplicon were associated with our variables of interest. Indeed, the investigated gene regions include sequences with distinct biological functions, such as transcription factor binding sites. This may explain why certain maternal stress variables, for instance PRAQ subscales, are associated with a specific CpG unit within the amplicon, but not others. Some CpG units that are significantly linked to maternal emotional stress or cortisol in our study are located within regulatory regions and CTCF or polycomb complex binding sites. Future studies are necessary to examine the functional significance of our findings.

Our study has some limitations that should be addressed. First, our study population of pregnant women is relatively small and does not entirely represent a random sample, since we noted a high socio-economic status and most women were highly educated. However, our study population shows medium levels of pregnancy-related anxiety and a normal prevalence of depressive symptoms measured by EDS score (Gaynes *et al.*, 2005). The lack of individuals with extreme measures of stress may explain the rather small regression coefficients of some of our findings and more pronounced effects might be detected in a population with more extreme depression or anxiety measures. However, despite the small sample size and lack of extreme measurements of stress, we did pick up on significant findings where other studies have also found associations (Chen *et al.*, 2014, Liu *et al.*, 2012). A recent genome-wide methylation study also clearly shows that the impact on fetal DNA methylation may be more widespread when assessing the association with non-medicated maternal depression or anxiety (Non *et al.*, 2014). Second, it is not possible to generalize our results for all ethnicities, as the population studied here was mainly European. Third, the methylation analyses in this study were carried out in cord blood peripheral blood mononuclear cells which is a mixture of cell types, mainly lymphocytes (T and B cells) and

monocytes. However, methylation of imprinted genes was shown to be comparable in the different cord blood cell fractions (Murphy *et al.*, 2012b). Lastly, we did not assess allele-specific methylation differences, since our main interest was to find DNA methylation differences in relation to prenatal stress. Although our effect sizes are too small to be due to loss/gain of imprinting, our results should therefore still be interpreted carefully.

In conclusion, we found that maternal emotional stress and cortisol during pregnancy are associated with fetal DNA methylation of IGF2 and GNAS. We found no evidence of an association between DNA methylation of these imprinted genes and child's ponderal index at birth. Taken together, our results add to the growing literature providing evidence that imprinted gene methylation is sensitive to prenatal adversity, whether it is exposure to toxins, famine or maternal stress.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors declare no conflicts of interest

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TABLES AND FIGURES

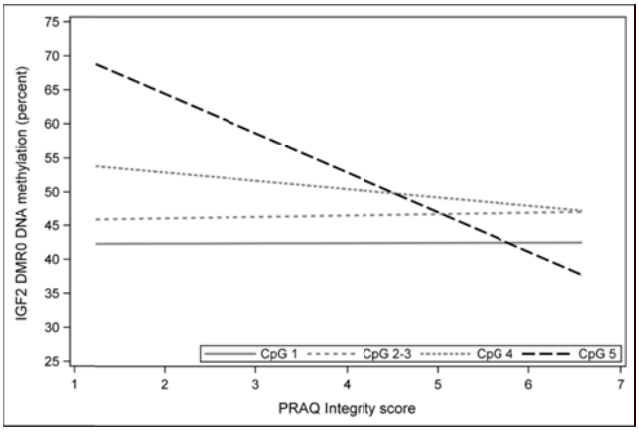

Figure 1: Relation between PRAQ-integrity and IGF2 DMR0 DNA methylation at each CpG unit. Light grey correlations are not significant. Black correlations remain significant after correction for multiple testing. PRAQ, pregnancy related anxiety questionnaire

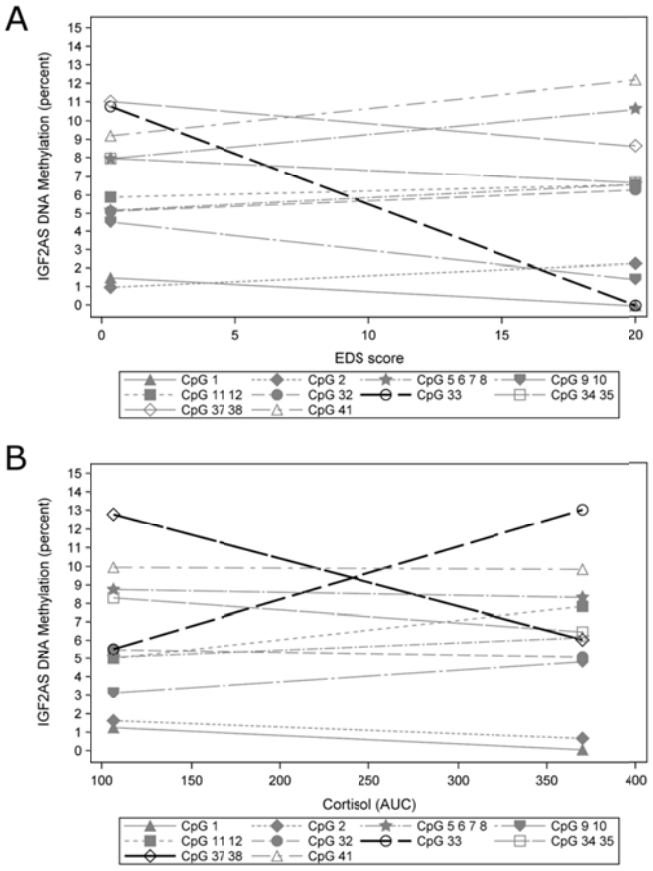

Figure 2: Relation between Edinburgh Depression Scale (EDS) scores (A) or cortisol area under the curve (AUC; B) and IGF2AS DNA methylation at each CpG unit. Light grey correlations are not significant. Black correlations remain significant after correction for multiple testing.

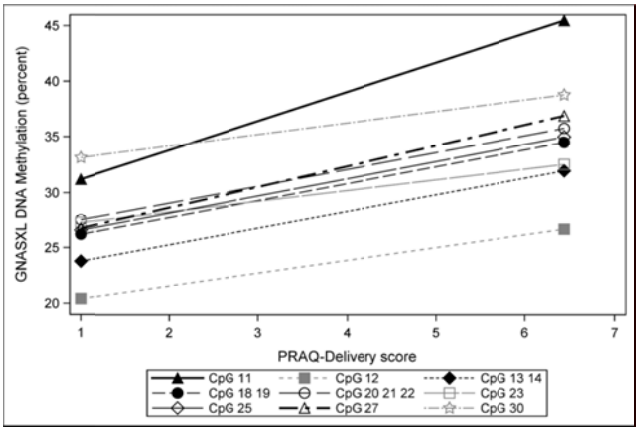

Figure 3: Relation between PRAQ-delivery scores and GNASXL DNA methylation at each CpG unit. Light grey correlations are not significant. Black correlations are significant, but only the association of CpG11 with PRAQ-delivery scores remains significant after correction for multiple testing. PRAQ, pregnancy related anxiety questionnaire

Effect	Location	β Estimate	Standard Error	DF	t value	P value
PRAQ-integrity	CpG1	0.000374	0.01372	147	0.03	0.9783
	CpG2-3	0.002059	0.01236	147	0.17	0.8679
	CpG4	-0.01220	0.01278	147	-0.95	0.3413
	CpG5	**-0.05809**	**0.01358**	**147**	**-4.28**	**<.0001***

Linear mixed model was corrected for maternal smoking during pregnancy, gestational age and gender of the baby. PRAQ, pregnancy-related anxiety questionnaire; DF, degrees of freedom. * remains significant after Holm's correction for multiple testing

Table 1: Results for the IGF2 DMR0 amplicon post-hoc tests to examine which CpG units have slopes unequal to zero.

Effect	Location	β estimate	Standard error	DF	t value	P value
EDS	CpG1	-0.00143	0.001385	603	-1.03	0.3032
	CpG2	0.000641	0.001213	603	0.53	0.5974
	CpG5-6-7-8	0.001348	0.001576	603	0.86	0.3927
	CpG9-10	-0.00157	0.001237	603	-1.27	0.2036
	CpG11-12	0.000344	0.001234	603	0.28	0.7805
	CpG32	0.000592	0.001271	603	0.47	0.6417
	CpG33	**-0.00556**	**0.001515**	**603**	**-3.67**	**0.0003***
	CpG34-35	-0.00068	0.001249	603	-0.54	0.5870
	CpG37-38	-0.00124	0.001433	603	-0.87	0.3860
	CpG41	0.001515	0.001248	603	1.21	0.2254
	CpG44-45	0.000716	0.001213	603	0.59	0.5552
cortisol	CpG1	-0.00005	0.000073	603	-0.62	0.5351
	CpG2	-0.00004	0.000069	603	-0.52	0.6002
	CpG5-6-7-8	-0.00002	0.000072	603	-0.22	0.8244
	CpG9-10	0.000064	0.000070	603	0.92	0.3563
	CpG11-12	0.000105	0.000070	603	1.52	0.1300
	CpG32	-0.00001	0.000071	603	-0.21	0.8362
	CpG33	**0.000287**	**0.000083**	**603**	**3.44**	**0.0006***
	CpG34-35	-0.00007	0.000070	603	-1.00	0.3182
	CpG37-38	**-0.00026**	**0.000073**	**603**	**-3.52**	**0.0005***
	CpG41	-0.000004	0.000070	603	-0.06	0.9543
	CpG44-45	0.000040	0.000069	603	0.58	0.5654

Linear mixed model was corrected for maternal smoking during pregnancy, gestational age and gender of the baby. EDS, Edinburgh depression scale; DF, degrees of freedom. * remains significant after Holm's correction for multiple testing

Table 2: Results for the IGF2AS amplicon post-hoc tests to examine which CpG units have slopes unequal to zero. Linear mixed model was corrected for maternal smoking during pregnancy, gestational age and gender of the baby. DF, degrees of freedom

Effect	Location	β Estimate	Standard error	DF	t value	P value
PRAQ-delivery	**CpG11**	**0.02619**	**0.007172**	**411**	**3.65**	**0.0003***
	CpG12	0.01152	0.007210	411	1.60	0.1107
	CpG13-14	**0.01504**	**0.007262**	**411**	**2.07**	**0.0390**
	CpG18-19	**0.01529**	**0.007208**	**411**	**2.12**	**0.0345**
	CpG20-21-22	**0.01521**	**0.007317**	**411**	**2.08**	**0.0382**
	CpG23	0.009639	0.007612	411	1.27	0.2061
	CpG25	**0.01552**	**0.007247**	**411**	**2.14**	**0.0328**
	CpG27	**0.01871**	**0.007244**	**411**	**2.58**	**0.0102**
	CpG30	0.01033	0.007245	411	1.43	0.1549

Linear mixed model was corrected for maternal smoking during pregnancy, gestational age and gender of the baby. PRAQ, pregnancy-related anxiety questionnaire; DF, degrees of freedom. Significant p-values are indicated in bold. * remains significant after Holm's correction for multiple testing

Table 3: Results for the GNASXL amplicon post-hoc tests to examine which CpG units have slopes unequal to zero.